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Short communication

Inoculation of *Sarcocystis neurona* merozoites into the central nervous system of horses

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Abstract

Equine protozoal myeloencephalitis (EPM) is a neurologic syndrome in horses from the Americas and is usually caused by infection with the apicomplexan parasite, *Sarcocystis neurona*. A horse model of EPM is needed to test the efficacy of chemotherapeutic agents and potential vaccines. Five horses that were negative for antibodies to *S. neurona* in their serum and cerebrospinal fluid (CSF) were injected in the subarachnoid space with living merozoites of the SN2 isolate of *S. neurona*. None of the horses developed clinical disease or died over a 132-day observation period. All five horses developed antibodies to *S. neurona* in their CSF and serum 3–4 weeks after injection. Two of the horses were examined at necropsy and no parasite induced lesions were observed in their tissues and no parasites were recovered from portions of their spinal cords inoculated on to cell cultures. Results of this study demonstrate that merozoites of

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the SN2 isolate of *S. neurona* will induce seroconversion but not clinical disease when inoculated directly into the CSF of nonimmune horses.

Keywords: *Sarcocystis neurona*; Equine protozoal myeloencephalitis; Experimental infection; Merozoites; Model

1. Introduction

Equine protozoal myeloencephalitis (EPM) is a neurologic syndrome in horses from the Americas and is usually caused by infection with the apicomplexan parasite, *Sarcocystis neurona* (Dubey et al., 1991). The condition was first recognized in the early 1970s (Beech and Dodd, 1974; Cusick et al., 1974; Dubey et al., 1974) and EPM has grown in importance over the subsequent decades. It is now considered the most important protozoal disease of horses in the Americas (MacKay et al., 1992). Serological surveys of horses from the United States, using the Western blot test, indicate that 45–53% of horses have antibodies to *S. neurona* (Bentz et al., 1997; Blythe et al., 1997; Salville et al., 1997) indicating that exposure to the parasite is high. Exposure of horses in Argentina and Brazil to *S. neurona* is estimated at 36% using the Western blot test (Dubey et al., 1999a,b). Sporocysts excreted by opossums (*Didelphis virginiana*) are the source of *S. neurona* infection (Dubey and Lindsay, 1998) and this explains why this disease is only observed in the Americas in the range of the opossum.

Fenger et al. (1997) reported experimental reproduction of clinical signs and lesions consistent with EPM in three of the five weanling horses inoculated with sporocysts obtained from opossums. *Sarcocystis neurona* was not observed in the tissues of any of the five inoculated horses but all five horses seroconverted in the Western blot test. Methods used to identify the sporocysts (Tanhauser et al., 1999) were not available at the time Fenger et al. (1997) conducted their study and a mixture of the three known species of *Sarcocystis* (*S. falcatula*; *S. neurona*; *S. speeri*) that use opossums as definitive hosts (Dubey and Lindsay, 1998, 1999) might have been present. Cutler et al. (1999) demonstrated that *S. falcatula* sporocysts from opossums were not infectious for horses.

A horse model of EPM is needed to test the efficacy of chemotherapeutic agents and potential vaccines. The present study was done to determine if *S. neurona* merozoites from cell cultures would develop and produce clinical EPM in horses when injected into the cerebrospinal fluid (CSF) at the subarachnoid space.

2. Materials and methods

2.1. Cell culture

Sarcocystis neurona merozoites (SN2 isolate) were grown and maintained in African green monkey (*Cercopithecus aethiops*) kidney cells (CV-1 cells, ATTC CCL-70, American Type Culture Collection, Rockville, MD, USA) as described previously (Lindsay et al., 1999). This isolate was obtained from a horse with EPM in 1990 (Davis et al., 1991) and

cryopreserved for 8 years until it was revived (Dubey and Lindsay, 1998). This isolate is pathogenic when inoculated into immunodeficient mice and for that reason it was chosen for use in this study. Additionally, results of PCR described by Tanhauser et al. (1999) confirmed the isolate as *S. neurona*. The same host cells and culture procedures were used to grow *S. falcatula* (Cornell isolate), *Neospora caninum* (NC-1 isolate), *N. hughesi* (AU-Nh1 isolate) and *Toxoplasma gondii* (RH isolate) for use in antibody testing.

For horse inoculations, SN2 isolate merozoites were harvested from infected cell cultures by removing the medium and replacing it with Hanks' balanced salt solution without calcium and magnesium (HBSS). The CV-1 cells were then removed from the plastic growth surface by use of a cell scraper. This cell mixture was passed through a 27-gauge needle attached to a 10 ml syringe to rupture host cells. The suspension was then filtered through a sterile 3 μm filter to remove cellular debris. After washing with HBSS, the merozoites were enumerated with a hemacytometer and resuspended in plasmalyte (Baxter, Deerfield, IL, USA). To determine if plasmalyte had an adverse effect on the merozoites, we incubated merozoites in plasmalyte at 4°C overnight and inoculated them direct on to CV-1 cells. Merozoites stored overnight at 4°C in plasmalyte were infectious for host cells. This indicated that plasmalyte would not adversely effect the merozoites used for inoculation into the CSF of horses.

Spinal cord tissue from two inoculated horses was removed from C4–C7 and homogenized in HBSS. The homogenate was inoculated onto CV-1 cells to grow and isolate *S. neurona*. The homogenate was removed 30 min post-inoculation (PI) and cell culture medium was added. The cultures were maintained for 60 days and examined for growth of *S. neurona* by inverted microscopy.

2.2. Horses

Six mixed breed horses and one mule between the ages of 2 and 17 years were obtained from the Department of Large Animal Surgery and Medicine, College of Veterinary Medicine, Auburn University, AL, USA (Table 1). They had been at the facility for 6 months to 1 year. They were on a regular deworming program, negative for equine infectious anemia, and vaccinated against tetanus, rabies, eastern and western viral encephalomyelitis, influenza, and rhinopneumonitis. The horses were clinically normal at the start of the study. Cerebrospinal fluid (CSF) was obtained from the lumbosacral space

Table 1
Animal, necropsy status, sex, age, infection status, and weights (kg)

Animal	Necropsy	Sex[a]	Age	Infect[b]	Start weight	End weight
Horse 1	Yes	F	17	Yes	468	477
Horse 2	Yes	F	17	Yes	443	468
Horse 3	No	F	7	Yes	473	477
Horse 4	No	G	12	Yes	520	534
Horse 5	No	G	4	Yes	582	605
Horse 6	No	F	8	No	459	445
Mule	Died	M	2	No	441	NA

[a] F: female; G: male neutered; M: male.
[b] Horses 1–4 received 5.0×10^6 and horse 5 received 5.0×10^5 merozoites.

2–8 months before the study and examined by Western blot for antibodies to *S. neurona*, *S. falcatula*, *T. gondii*, *N. caninum* and *N. hughesi*.

2.3. Inoculation of horses with S. neurona and examinations

On Day 0, the seven animals were assigned to inoculated (five) or control (two) groups. The mule was placed in the negative control group. Approximately 10 ml of CSF was collected from each animal under general anesthesia by atlantooccipital (A–O) puncture from the subarachnoid space with a spinal needle. After CSF removal, 5×10^6 *S. neurona* merozoites suspended in 10 ml plasmalyte were inoculated into the subarachnoid space of four horses (horses 1–4). A fifth horse (horse 5) received 5×10^5 merozoites suspended in 10 ml plasmalyte. The mule and control horse (horse 6) received 10 ml plasmalyte alone after removal of the CSF fluid. Western blot analysis of the Day 0 CSF samples (Granstrom et al., 1993) indicated that all seven animals were negative for *S. neurona*, *S. falcatula*, *T. gondii*, *N. caninum* and *N. hughesi* at the time of inoculation. Serum was collected from each animal on Days 0, 8, 12, 20, 30, 40, 53, 75, 84, and 102 PI and tested for *S. neurona* antibodies by ELISA (see ahead). CSF samples were obtained by the A–O route on Days 30, 56 and 120 PI. After inoculation, the horses were maintained as a group in a grass paddock with free choice hay and a complete pelleted ration (Nutrena Feed Division, Minneapolis, MN, USA) for 132 days. All animals were observed daily for any changes in clinical condition, behavior, appetite or gait. Neurological examinations were conducted weekly during the study. No medications were given during this period except for the anesthetic agents given by IM and IV routes at the time of CSF collections. Each animal was weighed on Days 0 and 112 (Table 1).

Horses 1 and 2 were euthanized 130 days PI and examined at necropsy (Table 1). The brain and spinal cord were removed. Portions of spinal cord were removed for parasite isolation and the remaining portions and several portions of the brain were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned and stained with hematoxylin and eosin for histological examination.

2.4. ELISA and Western blot serology

We developed an enzyme-linked immunoassay (ELISA) to measure antibody responses of the horses to experimental *S. neurona* infection. Lysates of purified *S. neurona* merozoites (1×10^5 parasites per well) were coated on to 96-well plates and served as antigen. Two-fold dilutions of serum samples (beginning at 1:10 and ending at 1:5120) were diluted in tris buffered saline (TBS). Serum samples were incubated for 1 h in the 96-welled plates and then the plates were washed three times using a plate washer. Bound antibodies recognizing *S. neurona* were detected with goat anti-horse antibodies conjugated with alkaline phosphatase (Sigma, St. Louis, Missouri, USA). Sigma 104 phosphatase substrate (*p*-nitrophenyl phosphate) was used to detect the presence of antibodies detected by an absorbance reading at 405 nm with a Tecan plate reader. The same *S. neurona* positive serum was utilized in each experiment to permit normalization of the samples. The negative cutoff was set at a 1/40 dilution of the serum.

The Western blot test described by Granstrom et al. (1993) was used to examine samples for antibodies to *S. neurona*.

3. Results

3.1. Clinical signs

Daily clinical examinations on the five inoculated horses did not demonstrate any abnormalities in behavior or appetite during the 132-day observation period. The gait of all inoculated horses was normal during the 132-day period except that horse 2 developed left fore limb lameness at 110 days PI. It was treated for a hoof abscess and the lameness resolved in 1 week. Neurological examinations on the five inoculated horses done at weekly intervals did not show any abnormalities. All five inoculated horses gained weight during the study (Table 1).

Clinical examination of control horse 6 was normal until Day 59 PI when a decline in body condition was noted. The horse was moved to an adjacent paddock and supplemented with hay and pelleted ration. The horse developed a crusting dermatitis due to dermatophilus and was treated with 5% benzoyl peroxide shampoo followed by iodine shampoo. Neurological findings were normal for horse 6 during the entire examination period during which time it lost 13.6 kg between Days 0 and 112. Clinical examination of the mule was normal until Day 58. The mule was depressed and moving stiffly. A swelling was noted in the right cervical region in the area where an intramuscular injection of xylazine had been given on Day 56 PI. Within 1 h the swelling had extended down the ventral thorax and to the left cervical region. The mule grew progressively ataxic and died the same day. A necropsy was conducted.

3.2. Immunological analysis

Results of ELISA testing are shown in Table 2. Positive titers were first observed 8 days PI in three of the five inoculated horses. Titers peaked in all five inoculated horses at 30 days PI. No titers were observed in the sera of negative control animals during the course of the study.

Western blot analysis of the CSF samples taken on Day 0 PI were negative. Samples of CSF examined at 1, 2 and 4 months PI were positive for *S. neurona* in all five inoculated horses. Reactivity of the 4-month CSF samples were weaker than at 1 or 2 months. The serum samples from inoculated horses were positive by 3 weeks PI (see Table 2). The control horse 6 became positive for *S. neurona* in the CSF by Western blot after 4 months indicating a natural exposure to the parasite from the environment.

3.3. Necropsy

Complete necropsies were performed on horses 1 and 2. Putative gross lesions were observed in the C4–C7 spinal cord segments, characterized by soft, friable gray matter in dorsal and ventral horns. These lesions were collected into transport medium to attempt isolation of the parasite. Adjacent, contiguous spinal cord was collected for histopathology.

Table 2
Reciprocal anti-*Sarcocystis neurona* ELISA titers[a]

DPI[b]	H1	H2	H3	H4	H5	H6	M
0	–	–	–	–	–	–	–
8	–	–	40	40	320	–	–
12	–	160	–	–	–	–	–
20	160	640	160	640	160	–	–
30	1280	1280	1280	>5120	>5120	–	–
40	640	160	160	80	160	–	–
53	–	–	–	–	–	–	–
75	–	–	–	–	–	–	NA
84	–	–	–	–	–	–	NA
102	–	–	–	–	–	–	NA

[a] H1: horse 1; H2: horse 2; H3: horse 3; H4: horse 4; H5: horse 5; H6: horse 6; m=mule; –: negative; NA: not applicable.
[b] Days post-inoculation.

Microscopic lesions were not observed in sections from contiguous areas of cervical spinal cord, cerebrum, brainstem, cerebellum, or cranial cervical, thoracic or lumbar spinal cord. No protozoa were isolated from portions of spinal cord. The mule was diagnosed as having a *Clostridium* sp. infection.

4. Discussion

We used direct inoculation of merozoites into the CSF in the present study because they can be grown in cell culture and their identity can be positively determined using PCR and ultrastructural examination (Lindsay et al., 1999; Tanhauser et al., 1999). We chose to inject merozoites into the CSF so they would avoid the necessary migrations needed if given intravenously or by another parenteral method. Horses in the present study remained clinically normal, but developed antibodies to *S. neurona* in their CSF. The lack of clinical disease in all inoculated horses and absence of lesions in the horses examined at necropsy, suggested that a low level infection occurred. The control horse 6 did seroconvert to *S. neurona* in the Western blot, but this was 4 months after inoculation of the five experimentally-infected horses and indicated natural exposure to *S. neurona*. Because the horses were kept on pasture it was impossible to prevent exposure to opossums.

The results of our study suggest that direct inoculation of *S. neurona* merozoites in to the CSF is not a viable animal model. Perhaps if the horses had been immunosuppressed prior to inoculation of merozoites and during the initial infection period they would have developed clinical EPM. Merozoite induced infections have been successful in nude mice (Marsh et al., 1997) and interferon-g knockout mice (Dubey and Lindsay, 1998; Dubey et al., 1999a,b; Lindsay et al., 2000). These rodent models maybe useful for examining the efficacy of chemotherapeutic agents and defining certain aspects of host immunity. However, because they are immunosuppressed these rodent models are not suitable for examining candidate vaccines.

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References

Beech, J., Dodd, D.C., 1974. Toxoplasma-like encephalitis in the horse. Vet. Pathol. 11, 87–96.

Bentz, B.G., Granstrom, D.E., Stamper, S., 1997. Seroprevalence of antibodies to *Sarcocystis neurona* in horses residing in a county of southeastern Pennsylvania. J. Am. Vet. Med. Assoc. 210, 517–518.

Blythe, L.L., Granstrom, D.E., Hansen, D.E., Walker, L.L., Bartlett, J., Stamper, S., 1997. Seroprevalence of antibodies to *Sarcocystis neurona* in horses residing in Oregon. J. Am. Vet. Med. Assoc. 210, 482–483.

Cusick, P.K., Sells, D.M., Hamilton, G.G., 1974. Toxoplasmosis in two horses. J. Am. Vet. Med. Assoc. 164, 77–80.

Cutler, T.J., MacKay, R.J., Ginn, P.E., Greiner, E.C., Porter, R., Yowell, C.A., Dame, J.B., 1999. Are *Sarcocystis neurona* and *Sarcocystis falcatula* synonymous? A horse infection challenge. J. Parasitol. 85, 301–305.

Davis, S.W., Daft, B.H., Dubey, J.P., 1991. *Sarcocystis neurona* cultured in vitro from a horse with equine protozoal myelitis. Equine Vet. J. 23, 315–317.

Dubey, J.P., Lindsay, D.S., 1998. Isolation of *Sarcocystis neurona* from possum (*Didelphis virginiana*) faeces in immunodeficient mice and its differentiation from *Sarcocystis falcatula*. Int. J. Parasitol. 29, 1823–1828.

Dubey, J.P., Lindsay, D.S., 1999. *Sarcocystis speeri* n. sp. (Protozoa: Sarcocystidae) from the opossum (*Didelphis virginiana*). J. Parasitol. 85, 903–909.

Dubey, J.P., Davis, G.W., Koestner, A., Kiryu, K., 1974. Equine encephalomyelitis due to a protozoan parasite resembling *Toxoplasma gondii*. J. Am. Vet. Med. Assoc. 165, 249–255.

Dubey, J.P., Davis, S.W., Speer, C.A., Bowman, D.D., De Lahunta, A., Granstrom, D.E., Topper, M.J., Hamir, A.N., Suter, M.M., 1991. *Sarcocystis neurona* n. sp. (Protozoa: Apicomplexa), the etiologic agent of equine protozoal myeloencephalitis. J. Parasitol. 77, 212–218.

Dubey, J.P., Venturini, M.C., Venturini, L., McKinney, J., Pecoraro, M., 1999a. Prevalence of antibodies to *Sarcocystis neurona*, *Toxoplasma gondii* and *Neospora caninum* in horses from Argentina. Vet. Parasitol. 86, 59–62.

Dubey, J.P., Kerber, C.E., Granstrom, D.E., 1999b. Serologic prevalence of *Sarcocystis neurona*, *Toxoplasma gondii* and *Neospora caninum* in horses in Brazil. J. Am. Vet. Med. Assoc. 215, 970–972.

Fenger, C.K., Granstrom, D.E., Gajadhar, A.A., Williams, N.M., McCrillis, S.A., Stamper, S., Langemeier, J.L., Dubey, J.P., 1997. Experimental induction of equine protozoal myeloencephalitis in horses using *Sarcocystis* sp. sporocysts from the opossum (*Didelphis virginiana*). Vet. Parasitol. 68, 199–213.

Granstrom, D.E., Dubey, J.P., Davis, S.W., Fayer, R., Fox, J.C., Poonacha, K.B., Giles, R.C., Comer, P.F., 1993. Equine protozoal myeloencephalitis: antigen analysis of cultured *Sarcocystis neurona* merozoites. J. Vet. Diagn. Invest. 5, 88–90.

Lindsay, D.S., Dubey, J.P., Horton, K.M., Bowman, D.D., 1999. Development of *Sarcocystis falcatula* in cell cultures demonstrates that it is different from *Sarcocystis neurona*. Parasitology 118, 227–233.

Lindsay, D.S., Thomas, N.J., Dubey, J.P., 2000. Isolation and characterization of *Sarcocystis neurona* from a Southern sea otter (*Enhydra lutris nereis*). Int. J. Parasitol. 30, 617–624.

MacKay, R.L., Davis, S.W., Dubey, J.P., 1992. Equine protozoal myeloencephalitis. Comp. Contin. (Ed.) Pract. Vet. 14, 1359–1366.

Marsh, A.E., Barr, B.C., Lakritz, J., Nordhausen, R., Madigan, R.E., Conrad, P.A., 1997. Experimental infection of nude mice as a model for *Sarcocystis neurona*-associated encephalitis. Parasitol. Res. 83, 706–711.

Salville, W.J., Reed, S.M., Granstrom, D.E., Hinchcliff, K.W., Kohn, C.W., Wittum, T.E., Stamper, S., 1997. Seroprevalence of antibodies to *Sarcocystis neurona* in horses residing in Ohio. J. Am. Vet. Med. Assoc. 210, 519–524.

Tanhauser, S.M., Yowell, C.A., Cutler, T.J., Greiner, E.C., MacKay, R.J., Dame, J.B., 1999. Multiple DNA markers differentiate *Sarcocystis neurona* and *Sarcocystis falcatula*. J. Parasitol. 85, 221–228.